Biotech Health Sci. 2014 August; 1(2): e22085.

Published online 2014 August 28. **Research Article**

Emergence of OXA-Type Extended-Spectrum β-Lactamases Among *Enterobacter cloacae* Isolates Collected From Hospitals of Tehran, Karaj and Qazvin, Iran

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Received: January 10, 2014; **Revised:** May 12, 2014; **Accepted:** July 1, 2014

Background: Extended-Spectrum β-Lactamases (ESBLs)-producing *Enterobacter cloacae* has been increasingly reported as a major clinical concern in recent years. TEM and SHV β-lactamase are the most common ESBL genotypes that are found in Enterobacteriaceae; however, there are also new families of ESBLs, including OXA-type enzymes, which are one of the most important mechanisms of resistance to oxyimino-cephalosporin antibiotics. OXA-type ESBLs are divided into five groups.

Objectives: The main aim of the present study was to determine the frequency of bla_{OXA} genes among ESBL-producing *E. cloacae* isolates in three distinct provinces of Iran.

Patients and Methods: A total of 82 non-repetitive ESBL-producing *E. cloacae* isolates were collected from hospitalized patient in Qazvin, Karaj, and Tehran hospitals, Iran. The isolates were identified by standard laboratory methods and then confirmed by API 20E strips. PCR and sequencing was performed for detection of bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-9}, and bla_{OXA-10} genes. The clonal relatedness of OXA-producing isolates was assessed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR).

Results: In total, 48 ESBL-producing isolates (58.5%) were positive for the bla_{OXA-1} gene. All bla_{OXA-1}-producing isolates showed multidrug resistant pattern. In this study, bla_{OXA-2}, bla_{OXA-9}, and bla_{OXA-10} genes were not detected. The ERIC-PCR results showed that 42 OXA-producing isolates (77.7%) were genetically diverse with different band patterns.

Conclusions: This study was the first report of the emergence of the plasmid-encoded bla_{OXA} genes among *E. cloacae* isolates in Iran. These findings highlight the need to use appropriate infection control policy and rational antibiotic therapy to reduce further spread of these resistant bacteria in the studied hospitals.

Keywords: Enterobacter cloacae; Extended-Spectrum β-Lactamases; OXA-type β-Lactamase

1. Background

Enterobacter cloacae is the most frequent bacterial that causes nosocomial infections among hospitalized patients (1, 2). This organism can cause several clinical diseases such as bacteremia as well as lower respiratory tract, skin, urinary tract, and soft-tissue infections (2). Risk factors associated with infections with *E. cloacae* include immunosuppression, long-term hospitalization, and invasive procedures or surgeries (3). Infection with *E. cloacae* is associated with increased morbidity and mortality in hospitalized patients, especially in intensive care units (ICUs) and other high-risk hospital settings (4). Beta-lactam compounds are important group of prescribed antibiotics for treatment of patients infected with *E. cloacae* (2); however, extensive and inappropriate use of broad-spectrum β-lactam antibiotics lead to appearance of multidrug resistant *E. cloacae* isolates, which severely limit the therapeutic options for treatment of infected patients. Although resistance of *Enterobacter* species to third-generation cephalosporins is most typically caused by overproduction of AmpC β-lactamases, the role of extended-spectrum β-Lactamases (ESBLs) has been increasingly reported among Enterobacteriaceae (5). ESBLs have been found mostly in *Klebsiella* species and *Escherichia coli* but have also been described in other Enterobacteriaceae including *Enterobacter*, *Citrobacter,* and *Serratia* species. ESBLs are the most important mechanisms of resistance to third-generation cephalosporins with remarkable ability to develop resistance to several classes of antimicrobial agents (6). These enzymes have been commonly located on plasmids that are transferable from strain to strain and between bacterial species. TEM and SHV are the most common types of ESBLs among *E. cloacae* (7); however, a number of different ESBL types have been recently identified in *Enterobacter* species such as CTX-M and OXA (8). There are five groups of OXA-type ESBLs.

The OXA group I includes OXA-5, 7, 10, and 13 and its extended spectrum derivatives (OXA-11, 14, 16, 17, and 19). Group II includes OXA-2, 3, 15, and 20. Group III includes OXA-1, 4, 30, and 31 whereas group IV and group V include only OXA-9 and LCR-1, respectively (9). OXA-type ESBLs, which belong to class D β-lactamases and are categorized into functional group 2d, are characterized by their hydrolytic activity against oxacillin and cloxacillin (10, 11). There are very limited epidemiologic data on the geographical distribution of OXA-type ESBLs-producing *Enterobacter* species (12).

2. Objectives

We aimed to assess the prevalence of OXA-type-ESBLs among clinical isolates of *E. cloacae* collected from Qazvin, Karaj, and Tehran Hospitals, Iran.

3. Patients and Methods

A total of 82 non-repetitive ESBL-producing *E. cloacae* isolates were collected from the patient admitted in different wards of Qazvin, Karaj, and Tehran hospitals. The bacterial isolates were identified by conventional laboratory techniques (13) and then confirmed by the API 20E (bioMerieux, France). The isolates were stored at -70℃ in trypticase soy broth containing 20% glycerol and were subcultured twice prior to testing. The bacterial isolates were recovered from different clinical specimens including tracheal aspirates, urine, sputum, blood, bronchial washings, wound, and cerebrospinal fluid. Forty-six patients (56.1%) were male and 36 (43.9%) were female. The mean age of the patients was 51.7±17.4 years (range, 17-83).The ESBL production was confirmed by phenotypic-combined disk method as recommended by Clinical and Laboratory Standards Institute (CLSI) guideline (14). Antibiotic discs were purchased from Mast (Mast Diagnostics Group Ltd, Merseyside, UK). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the quality control strains in antimicrobial susceptibility testing.

3.1. Detection of blaOXA Genes by Polymerase Chain Reaction and Sequencing Method

The bacteria were regularly subcultured on nutrient agar at 37℃ for 24 hours. Method of extracting genomic DNA from clinical *E. cloacae* isolates was boiling. In brief, four or five colonies of the overnight culture of each isolate were suspended in 300 μL of TE buffer (10 mM Tris-HCL, 1 mM EDTA, and pH = 8.0). The suspensions were heated for five minutes at 95℃ and then cooled down by placing it on ice. The cellular debris was removed by centrifugation at 12000 g for ten minutes. One microliter of supernatant was used as template DNA for polymerase chain reaction (PCR) method. The quantity and purity of DNA in the samples was measured by NanodropND-1000 (Nanodrop Technologies, Wilmington, USA). Appropriate precautions were taken to avoid cross-contamination during sample preparation. PCR assay was performed for the detection of β-lactamases OXA genes (bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-9}, and bla_{OXA-10} genes) using specific primers (Table 1). PCR amplifications were performed in a thermocycler (applied biosystems, Foster City, CA, USA) as follows: 95℃ for five minutes and 35 cycles of five minutes at 95℃, one minute at specific annealing temperature for each primer, and one minute at 72℃. A final extension step of ten minutes at 72℃ was performed. Amplification reactions were performed in a total volume of 25 μL (24 μL of PCR master mix plus 1 μL of template DNA) containing 5 ng of genomic DNA, 2 U of Taq DNA polymerase, 10 mM dNTP mix at a final concentration of 0.2 mM, 50 mM $MgCl_2$ at a final concentration of 1.5 mM, 1 μM of each primer, and 1X PCR buffer (final concentration). PCR products were electrophoresed on a 1% agarose gel at 100 V and stained with cyber green solution; finally, they were visualized in gel documentation system (UVtec, Avebury House, Cambridge CB4 1QB UK). The purified PCR products were sequenced by the Macrogen Company (Seoul, Korea) and the sequence alignment and analysis were performed online using the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

3.2. Enterobacterial Repetitive Consensus Polymerase Chain Reaction

The epidemiological association among OXA-producing *E. cloacae* isolates were analyzed by enterobacterial repetitive consensus PCR (ERIC-PCR) using the appropriate primers. Cycling conditions were as follows: three minutes at 95℃, 40 cycles of 30 seconds at 92℃, one minute at 40℃, eight minutes at 72℃, and final extension of 16 minutes at 72℃. The resulting products were analyzed on 1.5% agarose gels. Fingerprints were compared visually and the patterns differing by at least one amplification band were classified as different (15).

Table 1. Oligonucleotide Primers Used for Detection of bla_{OXA} Genes of *Enterobacter cloacae*

Name	Sequence (5'→3')
OXA-1F	ACA CAATACATATCAACTTCGC
OXA-1R	AGT GTGTTTAGAATGGTGATC
OXA-2F	TTC AAGCCA AAGGCACGATAG
OXA-2R	TCC GAGTTGACTGCCGGGTTG
OXA-9R	CGTCGCTCACCATATCTCCC
OXA-9R	CCTCTCGTGCTTTAGACCCG
OXA-10F	CGT GCTTTGTAAAAGTAGCAG
OXA-10R	CAT GATTTTGGTGGGAATGG

4. Results

In this study, the bacterial isolates were obtained from different clinical specimens including urine (30 isolates; 36.6%), wound (21 isolates; 25.6%), sputum (four isolates; 4.9%), bronchoalveolar lavage (two isolates; 2.4%), trachea (14 isolates; 17.1%), blood (ten isolates; 12.2%), and cerebrospinal fluid (one isolates; 1.2%). Isolates were obtained from 42 patients admitted to ICUs (51.2%), 16 to internal medicine (19.5%), seven to infectious diseases (8.5%), four to neurology (4.9%), eight surgery (9.8%), and five to orthopaedic (6.1%) wards. Antimicrobial susceptibility pattern showed that all ESBL-producing isolates had multidrug resistant pattern. In total, 48 ESBL-producing isolates (58.5%) had positive results for the bla_{OXA-1} gene. OXA-producing isolates showed multidrug resistance pattern, in which one (2.1%) and two (4.2%) OXA-1-producing isolates were resistance to imipenem and meropenem, respectively. OXA-1-producing isolates were mostly isolated from ICU (23 isolates; 47.9%) and internal medicine (seven isolates; 14.6%) wards, respectively. These isolates were mostly obtained from wound (16 isolates; 33.3%) and urine (four isolates; 26.7%) samples, respectively (Table 2). According to ERIC-PCR results, 42 OXA-producing isolates (77.7%) showed different genotypes with distinct ERIC-PCR patterns, indicating clear heterogeneity in their genetic profiles.

Table 2. Distribution of bla_{OXA-1}-Producing *Enterobacter cloacae* Isolates Regarding Clinical Source and Hospital Wards [a, b]

	Results
Hospital wards	
ICU	23 (47.9)
Neurology	4 (8.3)
Surgery	5 (10.4)
Infectious diseases	5 (10.4)
Orthopedic	4 (8.3)
Internal medicine	7 (14.6)
Total	48 (100)
Clinical specimens	
Urine	11 (22.9)
Wound	16 (33.3)
Trachea	10 (20.8)
Blood	7 (14.6)
Sputum	2 (4.2)
CSF	1 (2.1)
BAL	1 (2.1)
Total	48 (100)

[a] Abbreviations: ICU, intensive care unit; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage.
[b] Data are presented as No. (%).

5. Discussion

Enterobacter cloacae has been increasingly identified as a cause of serious nosocomial infections. In recent years, ESBL production among these isolates is becoming a major clinical concern because of its ability to develop resistance to several classes of antimicrobial agents and high potential for transmission of resistance to other bacterial species (16). Clinical microbiology laboratories play a vital role in the detection and control of ESBL-producing organisms (17); however, many laboratories are not fully aware of the importance of ESBL-producing organisms and how to detect them effectively. ESBL presence is not routinely tested in most laboratories in Iran. OXA-type enzymes are clinically relevant class D β-lactamases, which are distributed mostly in gram-negative pathogens such as Enterobacteriaceae, *Pseudomonas* species, and *Acinetobacter* species. There are little data on the prevalence of OXA genes among ESBL-producing *E. cloacae* around the world (10, 11). In the present study, 58.5% of ESBL-producing *E. cloacae* isolates carried bla_{OXA-1} genes. To the best of our knowledge, this is the first report of the emergence of the bla_{OXA}genes-harboring *E. cloacae* in Iran. TEM-type and SHV-type ESBLs were the most co mMon genes among clinical isolates of Enterobacteriaceae in Iran. In a study, Shahcheraghi et al. reported 39 (69.6%) and 18 (32.1%) of ESBL-producing *Klebsiella* pneumonia isolates containing bla_{SHV} and bla_{TEM}, respectively (18). In another study from Tehran, Mirsalehian et al. showed presence of SHV and TEM among the ESBL-producing *E. cloacae* isolates (19). In total, prevalence rate of bla_{OXA} genes in *E. cloacae* isolates varies widely by country. In a study from southern Iran, Mostatabi et al. reported that 20.51% of ESBL-producing *Serratia* isolates carried bla_{OXA} gene (20). In a study from Tunisia, Bourouis et al. showed presence of bla_{OXA-1} genes among ESBL-producing *E. cloacae* (21). In Cameroon, Lonchel et al. showed that bla_{OXA-1} genes were present in all of ESBL-producing *E. cloacae* isolates (22). In a study from Madagascar, Rakotonirina et al. reported that 14.28% of ESBL-producing *E. cloacae* isolates harbored bla_{OXA-1} gene, which was lower than our findings (23). In another study from North-Lebanon, Sana et al. showed that 45.2% of ESBLs-producing *E. coli* isolates carried bla_{OXA} gene (24). In the present study, most bla_{OXA-1}-producing *E. cloacae* isolates were frequently collected from the patients admitted to ICUs. The ICU stay, exposure to third-generation cephalosporins, and the use of invasive procedures such as urinary catheterization might predispose these patients to infections with these resistant organisms. In this study, all bla_{OXA-1}-producing isolates showed multidrug resistance pattern. The highest sensitivity rate was against meropenem and imipenem. However, we encountered a low rate of carbapenem resistance among OXA-positive *E. cloacae* isolates, which could be important if these isolates become more prevalent in the future. ERIC-PCR analysis showed that more than 75% of isolates with bla_{OXA-1} were epidemiologically unrelated; it suggests that the dissemination of these isolates was not due to a

clonal outbreak. It could be explained by the fact that the isolates in the present study were collected from different hospitals in three different regions in Iran. This study demonstrated the presence of bla_{OXA} genes in *E. cloacae* isolates in Iran for the first time. We also found multidrug resistance pattern in these isolates. The high prevalence rate of OXA-type ESBLs in this study represents a serious problem in our hospital settings, which can be circumvented only through early detection and stringent control of these resistant organisms.Careful monitoring and use of appropriate infection control policy are necessary in preventing further emergence and spread of resistant organisms in our hospitals.

Acknowledgements

We express our sincere gratitude to Cellular and Molecular Research Center, Qazvin University of Medical Sciences, for providing study grants.

Author's Contributions

Conception and design of the study, Amir Peymani; laboratory work, Amir Peymani, Marzieh Marandi, Mehdi Sahmani and Mahdi Mohammadi Ghanbarlou; data analysis and interpretation, Taghi Naserpour Farivar and Reza Najafipour.

Funding/Support

This study was financially supported by the Cellular and Molecular Research Center and Research Deputy of Qazvin University of Medical Sciences (grant number: 90/437), Qazvin, Iran.

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